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Video Article

Analysis of Developing Tooth Germ Innervation Using Microfluidic Co-culture Devices

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Abstract

Innervation plays a key role in the development, homeostasis and regeneration of organs and tissues. However, the mechanisms underlying these phenomena are not well understood yet. In particular, the role of innervation in tooth development and regeneration is neglected.

Several *in vivo* studies have provided important information about the patterns of innervation of dental tissues during development and repair processes of various animal models. However, most of these approaches are not optimal to highlight the molecular basis of the interactions between nerve fibres and target organs and tissues.

Co-cultures constitute a valuable method to investigate and manipulate the interactions between nerve fibres and teeth in a controlled and isolated environment. In the last decades, conventional co-cultures using the same culture medium have been performed for very short periods (e.g., two days) to investigate the attractive or repulsive effects of developing oral and dental tissues on sensory nerve fibres. However, extension of the culture period is required to investigate the effects of innervation on tooth morphogenesis and cytodifferentiation.

Microfluidics systems allow co-cultures of neurons and different cell types in their appropriate culture media. We have recently demonstrated that trigeminal ganglia (TG) and teeth are able to survive for a long period of time when co-cultured in microfluidic devices, and that they maintain in these conditions the same innervation pattern that they show *in vivo*.

On this basis, we describe how to isolate and co-culture developing trigeminal ganglia and tooth germs in a microfluidic co-culture system. This protocol describes a simple and flexible way to co-culture ganglia/nerves and target tissues and to study the roles of specific molecules on such interactions in a controlled and isolated environment.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53114/>

Introduction

Innervation plays a key role in the development, homeostasis and regeneration of organs and tissues^{1,2}. Furthermore, innervation is involved in the regulation of stem cell proliferation, mobilization and differentiation³⁻⁵. Indeed, recent studies realised in tissues of the orofacial complex have shown that parasympathetic nerves are necessary for epithelial progenitor cells function during the development and regeneration of the salivary glands^{6,7}. Similarly, it has been demonstrated that innervation is necessary for the development and maintenance of taste buds⁸⁻¹¹. Thus, it is important to analyse the yet neglected roles of innervation in the development of other important orofacial organs and tissues such as teeth.

In spite of the rich innervation of adult teeth, and in contrast to all other organs and tissues of the body, developing teeth start to be innervated at the earliest postnatal stages. Teeth develop as a result of sequential and reciprocal interactions between the oral ectoderm and cranial neural crest-derived mesenchyme. These interactions give rise to epithelial-derived ameloblasts and mesenchyme-derived odontoblasts that are responsible for the formation of enamel and dentin, respectively¹². Sensory nerves from the trigeminal ganglia and sympathetic nerves from the superior cervical ganglia innervate the adult teeth¹³⁻¹⁵. During embryogenesis, nerve fibres emanating from the trigeminal ganglia project towards the developing tooth germs and progressively surround them but they do not penetrate into the dental papilla mesenchyme¹³. Nerve fibres enter the dental pulp mesenchyme at more advanced developmental stages that correlate with odontoblast differentiation and dentin matrix deposition¹⁶. Dental pulp innervation is completed soon after tooth eruption in the oral cavity¹³. Previous studies have revealed that various semaphorins and neurotrophins are involved in the regulation of innervation during odontogenesis¹⁶⁻¹⁹. Earlier studies have clearly demonstrated that innervation is a prerequisite for tooth formation in fishes²⁰. More recent studies have shown that homeostasis of dental mesenchyme stem cells in mouse incisors is regulated by sensory nerves via secretion of sonic hedgehog (shh)²¹. Nevertheless, the role of innervation in tooth initiation, development and regeneration is still highly controversial in mammals²²⁻²⁴.

A plethora of *in vivo* studies have provided important information about the patterns of innervation of dental tissues during development and repair processes of various animal models^{13,25,26}. However, most of these approaches are not optimal to highlight the molecular basis of the interactions between nerve fibres and target organs and tissues. Co-cultures constitute a valuable method to investigate and manipulate the interactions between nerve fibres and teeth in a controlled and isolated environment^{26–29}. At the same time, co-culturing is subject to various technical adjustments. For example, nerves and specific dental tissues (e.g., dental pulp, dental follicle, dental epithelium) often require different culture media in order to guarantee tissue survival for long periods of time^{30–32}.

In the last decades, conventional co-cultures using the same culture medium have been performed for very short periods (e.g., two days) to investigate the attractive or repulsive effects of developing oral and dental tissues on sensory nerve fibres^{27–29}. However, extension of the culture period is required to investigate the effects of innervation on tooth morphogenesis and cytodifferentiation, and to study the dynamics of nerve fibres branching within target organs. Therefore, non-contiguous co-cultures would be more suitable to perform studies on neuronal-dental tissues interactions.

Microfluidics systems allow co-cultures of neurons and different cell types in their appropriate culture media. In these devices, dental tissues and neurons are separated in different compartments, while allowing the growth of axons from the neural cell bodies through microchannels towards the compartment containing their target tissue³³. Microfluidic devices have been already used to study the interactions between neurons and microglia^{34,35}, as well as cell to cell interactions in cancer and neovascularization³⁵. Moreover, these systems have been used to study the interactions between dorsal root ganglia and osteoblasts³⁶.

We have recently demonstrated that trigeminal ganglia (TG) and teeth are able to survive for long periods of time when co-cultured in microfluidic devices³⁷. Moreover, we have demonstrated that teeth from different developmental stages maintain in these *in vitro* conditions the same repulsive or attractive effects on trigeminal innervation that they show *in vivo*³⁷. This protocol provides information about a simple, powerful and flexible way to co-culture ganglia/nerves and target tissues and to study the roles of specific molecules on such interactions in a controlled and isolated environment.

Protocol

All mice were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary office, Zurich.

1. Preparation of Dissection Material, Culture Media, Microfluidic Devices

1. Autoclave micro-dissection forceps and scissors (121 °C, sterilization time: 20 min) and store them in a sterile container.
2. Sterilize glass coverslips (24 mm x 24 mm) by incubating them in 1 M HCl for 24 hr on an orbital shaker at 37 °C. Wash them three times with sterile, distilled H₂O and three times with ethanol 99%. Dry then the coverslips at 37 °C or under sterile flow hood. Finally, autoclave or expose the coverslips to UV light (30 min) to complete the sterilization. Coverslips can then be stored in ethanol 70%.
3. Remove carefully the AXIS Axon Isolation Devices from the package using sterile forceps and place them in a sterile Petri dish.
4. Using a sterile biopsy punch (ø: 1mm) create one hole per sample to be cultured (**Figure 1**) in correspondence of the culture chambers. NOTE: Do not punch too close to the microgrooves, as they might be damaged by the pressure applied.
5. Sterilize the AXIS Axon Isolation Devices by immersing them in ethanol 70%. Dry then AXIS Axon Isolation Devices and coverslips completely under a sterile flow hood. Wait a minimum of 3 hr before proceeding.
NOTE: incomplete drying will result in defective assembly of the microfluidic devices.
6. Place each coverslip into a 35 mm Petri dish or into a well within a 6-wells plate.
7. Place the AXIS Axon Isolation Device onto the coverslip and press gently but firmly with a forceps with bent ends in order to allow full adhesion between the isolation device and the glass coverslip.
8. In each culture chamber, pipette 150 µl of poly-D-lysine (0.1 mg/ml in sterile, distilled H₂O). Place the microfluidic devices under vacuum for 5 min, in order to remove all the air from the culture chambers.
9. If air can still be seen within the chambers, re-pipette the poly-D-lysine solution into the chambers.
10. Incubate the devices with poly-D-lysine O/N at 37 °C.
11. Wash chambers three time with sterile, distilled H₂O.
12. Fill chambers with 150 µl laminin working solution (Sigma-Aldrich, 5 µg/ml, in PBS or serum-free medium), and incubate O/N at 37 °C.
13. Prepare 50 ml of medium for trigeminal ganglia cultures³⁷, composed as follows: 48 ml Neurobasal medium, 1 ml B-27, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 5 ng/ml nerve growth factor (NGF), 0.25 pM cytosine arabinoside.
14. Prepare 50 ml of medium for tooth germ cultures³⁷, composed as follows: 40 ml DMEM-F12, 10 ml foetal bovine serum (FBS, final concentration: 20%), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 150 µg/ml ascorbic acid.

2. Mouse Embryo Generation and Dissection

1. Determine embryonic age according to vaginal plug (vaginal plug: embryonic day of development 0.5, E0.5) and confirm it via morphological criteria. For this protocol, we generally use E14.5-E17.5 mouse embryos.
2. Clean the dissection area and the stereoscope with ethanol 70%.
3. Sacrifice the pregnant mother via cervical dislocation. Block the neck of the mouse with the first and second finger onto a grid, and pull with decision the tail.
4. Dissect the skin around the lower abdomen, and open the abdomen using scissors. Locate the uterus: during such late stages of pregnancy, the uterus abundantly fill the abdominal cavity.
5. Dissect out the uterus and place in a tube filled with PBS on ice. When on ice, the tissue can be left for several hr. Discard the corpse of the mother according to institutional guidelines
6. Dissect out the embryos from the uterus and free them from their extraembryonic tissues. Place the embryos in PBS on ice.

7. Decapitate the embryos using scissors, and separate the lower jaw from the rest of the head using micro-dissection scissors (**Figure 2A**). Remove precisely the lower jaw, without damaging the trigeminal ganglia; as the latter are localized in close proximity to the lower jaw, their accidental damage is possible. Preserve the lower jaw and the rest of the head in cold PBS, on ice.
8. To dissect TG, take the head and place it onto a dissection glass Petri dish, previously filled with cold PBS. Using the forceps, remove the skin and the skull. Remove then the telencephalon and the cerebellum by placing forceps below the telencephalon and lift; the telencephalon and the cerebellum will flip together, leaving the bottom of the skull exposed.
9. Localize the trigeminal ganglia (shown in **Figure 2B**). Use the forceps to separate the TG from the trigeminal nerves. Eliminate the remnants of the trigeminal projections using the dissection needles as knives. Place the dissected TG in a Petri dish filled with cold PBS and keep them on ice.
10. To dissect embryonic teeth, place the lower jaw, previously separated from the skull, onto the dissection glass Petri dish, filled with cold PBS. Using dissection needles as knives, remove the tongue and the skin surrounding the jaw. Separate the left and the right hemi-jaws by cutting along the midline of the jaw. The tooth germs are easily visible, as shown in **Figure 1C**. Isolate the tooth germs using dissection needles and remove the excess of non-dental tissues. Place the dissected tooth germs in a Petri dish filled with cold PBS and keep them on ice.

3. Microfluidic Co-cultures

1. After dissection, remove laminin from the microfluidic devices. Fill the chambers with 200 μ l of the respective media.
2. With forceps, transfer gently the dissected TG and tooth germs into the holes created by punching (**Figure 1D**). Make sure that the tooth germs do not float and that they sink until they contact the coverslips.
3. Culture the samples in incubator at 37 $^{\circ}$ C, 5% CO₂.
4. Change the culture medium every 48 hr. Do not empty the chambers completely, and do not pipette directly into the culture chambers. Complete emptying of chambers would result in the formation of air bubbles within the chambers; direct pipetting into the chamber would result in axonal damage. To avoid these issues, remove the medium pointing the pipette towards the external side of the wells; similarly, pipette the fresh medium on the side of the wells located opposite to the chambers.
5. During the culture period, co-cultures can be easily imaged by time-lapse microscopy. Co-cultures can be maintained for over 10 days.
6. After the culture period, wash the chambers by pipetting 150 μ l of PBS into one well per chamber, and letting PBS flow through the chambers three times.
7. Remove the PBS and fix the samples by pipetting 150 μ l of paraformaldehyde 4% (in PBS) in one well per chamber; incubate at RT for 15 min.
8. Wash the chambers twice with PBS as described in 3.6.
9. Proceed with further analysis.

Representative Results

These results show that isolated trigeminal ganglia can grow in one compartment of the microfluidic device and, in addition, that the development of the isolated tooth germs is sustained for a long period of time in the other compartment of the microfluidic device. Different culture media are used in the two compartments, and the microgrooves between the two compartments allow extension of axon from the trigeminal ganglion towards the developing tooth germs. **Figure 3** represents a visualization of neurofilament via immunofluorescence³⁷, in a co-culture of a mouse embryonic trigeminal ganglion and mouse embryonic incisor in the described microfluidic co-culture system. Mouse incisors are not innervated during development *in vivo*; consistently, axons from the trigeminal ganglion are repelled by the developing tooth germ in culture for as much as 10 days. **Figure 3A** shows an overview of the axonal chamber in this co-culture system. **Figure 3B** and **3C** show progressive magnifications of the neurites within the axonal chambers and the micro-groves. Tooth germs (or any co-cultures organs or tissues) can be easily removed from the microfluidic devices and analysed separately *e.g.*, processed for histological stainings or for gene expression analysis.

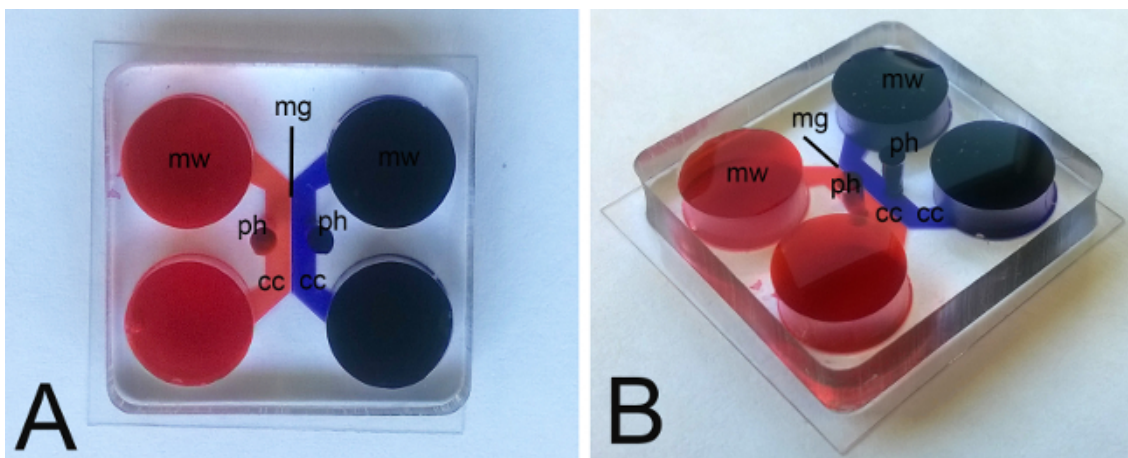


Figure 1. Views of mounted microfluidic co-culture device (A) from above; (B) partially lateral. The culture chambers (cc) are highlighted in red (eosin) and blue (toluidine blue); microgrooves (mg) can be seen as a white line between the culture chambers. Ganglia and co-cultured organs/tissues are placed into the appropriate culture chamber via the punched holes (ph) in the device. Media are added and removed via the wells (mw). [Please click here to view a larger version of this figure.](#)

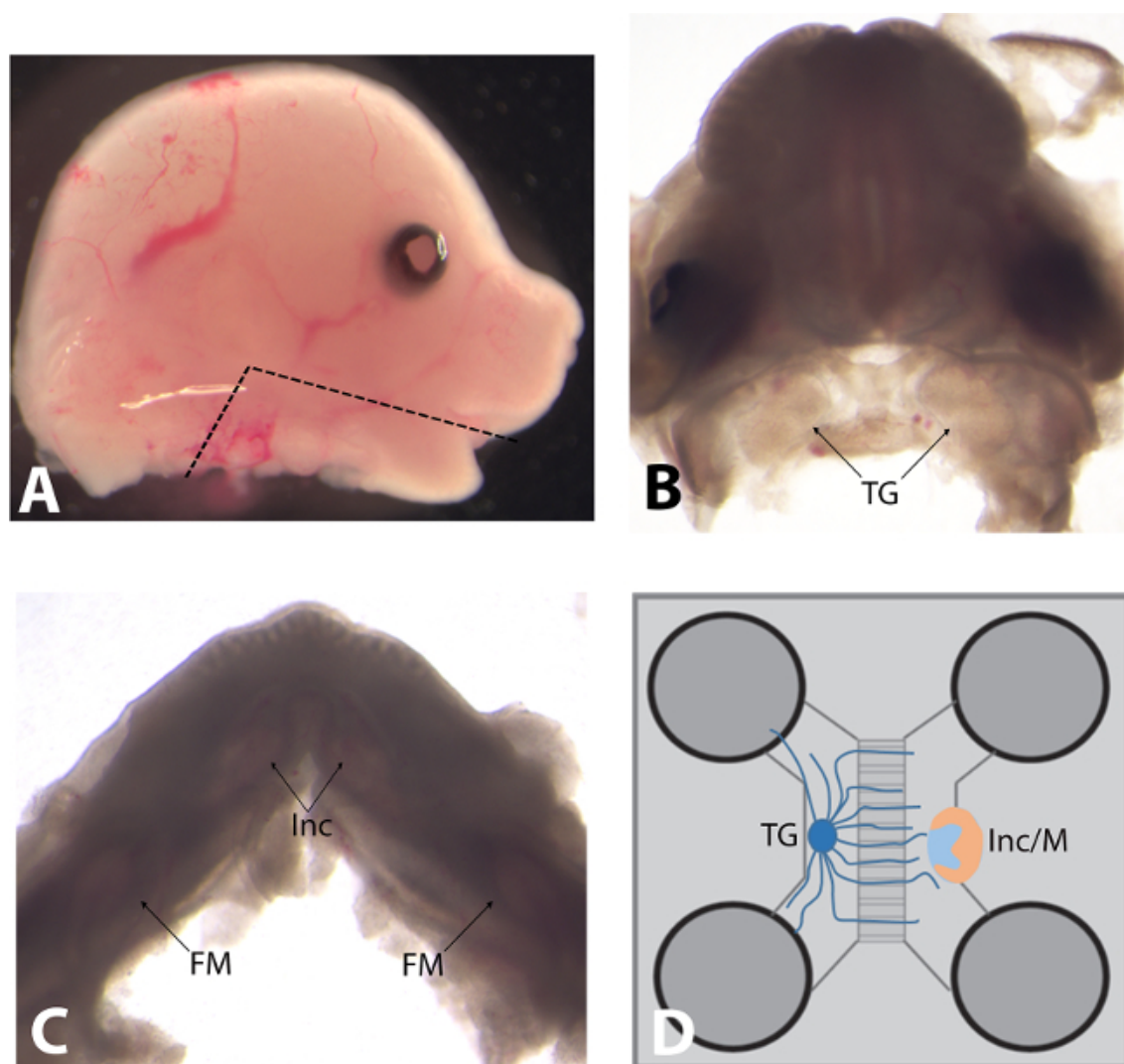


Figure 2. (A) Lateral view of a mouse embryo head (embryonic day of development E14.5). The black line indicates where the lower jaw should be cut in order to preserve integrity of both trigeminal ganglia and tooth germs. (B) Mouse embryo head (E14.5) after removal of lower jaw, skull and telencephalon. Black arrows indicate trigeminal ganglia (TG). (C) Lower jaw of mouse embryo (E14.5) after removal of the tongue. Black arrows indicate the localization of the different tooth germs (Inc: incisor; FM: first molars). (D) Schematic representation of the microfluidic co-culture device. [Please click here to view a larger version of this figure.](#)

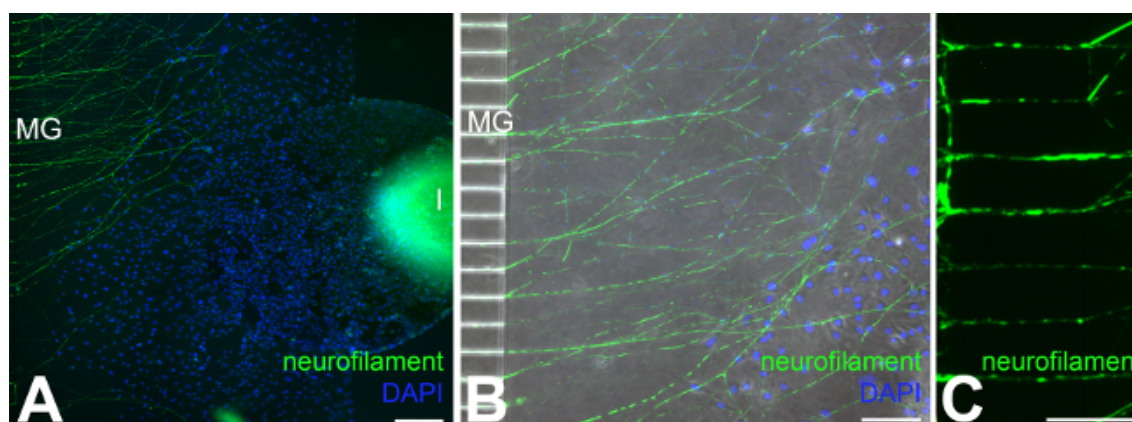


Figure 3. Co-culture of trigeminal ganglion and incisor tooth germ from wild-type E15.5 mouse embryos. (A) Overview of the axonal chamber (neurofilament, DAPI). Scale bar: 300 µm. (B) Higher magnification of the microgrooves and neurites growing into the axonal chamber (neurofilament, DAPI; superposition of fluorescent and brightfield images). Scale bar: 150 µm. (C) Higher magnification showing the growth of the axons within the microgrooves (neurofilament). Scale bar: 75 µm. [Please click here to view a larger version of this figure.](#)

Discussion

Previous *in vitro* studies of tooth innervation were based on conventional co-cultures of trigeminal ganglia and dental tissues or cells^{26,28,29}. These studies were conducted to investigate mainly the attractive effects of these cells or tissues on sensory axons³⁸. Although bringing significant advances in the field, several technical issues were raised. Tooth germs start to degenerate after few days of culture³⁷. Based on these observations, growing neurons and teeth in the same culture conditions impair any eventual analysis of molecules involved in the cross talk between these two tissues. Optimal culture conditions are needed to preserve the physiological molecular profile of trigeminal ganglia and dental tissues.

Microfluidics systems have been used so far to co-culture neurons and various cell types in optimized media^{34,36}. This microfluidics system can represent more faithfully the *in vivo* situation, where neural cell bodies, axonal terminals and target tissues are generally exposed to different cellular and molecular microenvironments. More recently, microfluidics devices have been used to co-culture whole dorsal root ganglia and osteoblasts³⁶. We recently demonstrated that trigeminal ganglia and teeth are able to survive for long periods of time when co-cultured in microfluidic devices³⁷. Moreover, we demonstrated that teeth from different developmental stages maintain in these *in vitro* conditions the same repulsive or attractive effects on trigeminal innervation that they exhibited *in vivo*³⁷.

The protocol described requires practice and manual dexterity, in particular for the microdissection of trigeminal ganglia and tooth germs. Trigeminal ganglia are easily torn during dissection. Therefore, we suggest the use of dissection needles instead of dissection forceps, in order to remove the tissue surrounding the ganglia. Similarly, particular care should be taken while dissecting tooth germs, in order to avoid damaging the tooth epithelium while separating it from the surrounding mesenchymal tissues. Furthermore, during the first day of co-culture, particular care should be taken in handling the incubator, as excessive vibration can impair trigeminal ganglion adhesion to the culture coverslip. During the co-culture period, media should be removed and added by pipetting into the wells, on the side opposite to the culture chambers. Aspiring or pipetting media in proximity of the culture chambers would result in axonal damage.

The protocol described can be modified to study the innervation of several embryonic organs and embryonic or postnatal tissues and cell types. Microfluidics systems could represent an appropriate platform to allow longer culture periods for the study of interactions between neurons and growing teeth. Moreover, separation of the neuronal from the dental compartment permits analysis of the effects of specific protein localization and quantification^{33,37}. Blocking antibodies or recombinant proteins could also be added to the separate compartments of the microfluidics devices for analysing their effects on neuronal and dental tissues. For example, treatment of trigeminal ganglia with NGF supports their survival and allows neuronal outgrowth. However, in this work exogenous NGF is absent from the tooth germ compartment, where tooth-derived signals are the only responsible for neuronal attraction or repulsion. Similarly, other recombinant molecules, antibodies or drugs can be used to manipulate the system in controlled conditions.

The major limitations of the protocol presented reside in the relative high cost of commercial microfluidic devices and the essentially 2D-set up of the co-culture system. In fact, although whole organs can be cultured, both organs and axons grow in 2D-adherent culture conditions on a glass coverslip; customization of the system would be needed in order to obtain a tridimensional, realistic representation of innervation.

In conclusion, microfluidic devices are optimal for investigating the role of innervation in developing or regenerating organs and permit the study of interactions between neuronal and target tissues in optimal culture conditions.

Disclosures

The authors declare that they have no competing financial interests.

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